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Short communication

Enzyme-free signal amplification for electrochemical detection of *Mycobacterium* lipoarabinomannan antibody on a disposable chip

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ABSTRACT

A simple, rapid, and disposable immunosensor at screen printed carbon electrode (SPCE) was developed by using gold nanoparticles (AuNPs) labeled Staphylococcal protein A (Au-SPA) as the electrochemical tag for detection of lipoarabinomannan antibody (anti-LAM). The immunosensor as the disposable chip was prepared by immobilizing capture antigen on screen printed carbon working electrode by passive adsorption, and characterized with scanning electron microscopy. After binding with the anti-LAM for further capture of Au-SPA, AuNPs were introduced as an electrochemical tag by the eletrooxidation of AuNPs in 0.1 M HCl to produce strong electroactive substance for signal amplification. Compared with the enzyme-based immunosensor, AuNPs as enzyme-free tag for signal amplification exhibited many advantages such as no requirement of deoxygenation, and high stability. Under optimal detection conditions and at a preoxidation potential of +1.3 V for 30 s, this method achieved the linear concentration of anti-LAM from 15.6 to 1000 ng mL^{-1} with a detection limit of 5.3 ng mL^{-1}. The immunosensor showed a good performance with high selectivity, acceptable stability, and simple operation, providing a promising application as an adjunctive tool in early tuberculosis diagnosis.

1. Introduction

Tuberculosis (TB), as an infectious disease caused by the bacillus *Mycobacterium tuberculosis* (MTB), is one of the greatest threats to the human health (Dye and Williams, 2010). In 2010, the World Health Organization (WHO) estimated that there were around 8.8 million incident cases of TB, 1.1 million deaths from TB among HIV-negative people and an additional 0.35 million deaths from HIV-associated TB (World Health Organization, 2011). The conventional methods, including sputum smear microscopy, solid culture and chest radiology (Steingart et al., 2006; Urbanczik, 1985), QuantiFERON-TB Gold test (Herrera et al., 2011), and Xpert MTB/resistance to rifampin (Boehme et al., 2010; Rachow et al., 2011), are efficient to reduce the mortality rate for the diagnosis of TB, but the complexity and high-cost assays limit their accessibility and effect. Unfortunately, TB is a disease of poverty and over 90% of the worldwide burden of TB takes place in low-income and middle-income countries. Therefore, it is necessary to build a cheap chip for rapid and accurate diagnosis of TB.

Alternatively, for development of TB assays, electrochemical techniques offered a promising opportunity due to the unique properties of rapidity, sensitivity, easy miniaturization and operation, and low cost. An enzymatic voltammetric immunosensor was developed for the determination of MTB antigen Ag231 with detection limit of 1.0 ng mL^{-1} (Díaz-González et al., 2005). Other TB-related antigens, such as 38 kDa, MPT51, ESAT-6, CFP-10, and lipoarabinomannan (LAM) had been employed for TB diagnosis (Verma and Jain, 2007). One promising antigen biomarker of TB is LAM, a 17.5 kDa polysaccharide antigen which constitutes 40% of the cell wall of mycobacteria (Brennan, 1995; Chatterjee and Khoo, 1998; Hunter et al., 1986; Schmidt et al., 2011). LAM is a potentially useful antigen in its acylated state, since it can achieve a high degree of specificity and sensitivity for the serodiagnosis of TB. Various tests have been developed to detect LAM in serum (Sada et al., 1992), pleural effusion (Dheda et al., 2009), sputum (Pereira Arias-Bouda et al., 2000) or urine (Boehme et al., 2005; Reither et al., 2009; Shah et al., 2010) in TB patients using ELISA for antigen recognition.

On the other hand, LAM can stimulate the human body to produce anti-LAM, which affords another efficient target for the application in the serodiagnosis of TB (Julián et al., 1997; Raja et al., 2008; Sada et al., 1990; Ben Selma et al., 2010; Steingart et al., 2009). The detection of antibodies in clinical specimens is becoming more attractive due to the advantages of strong antibody response during the infection of TB, no requirement of living cells, and noninvasion. Here, a simple, rapid, and disposable immunosensor at screen printed carbon electrode (SPCE) was developed using gold nanoparticles (AuNPs) labeled with Staphylococal protein A (Au-SPA) as the electrochemical tag to measure

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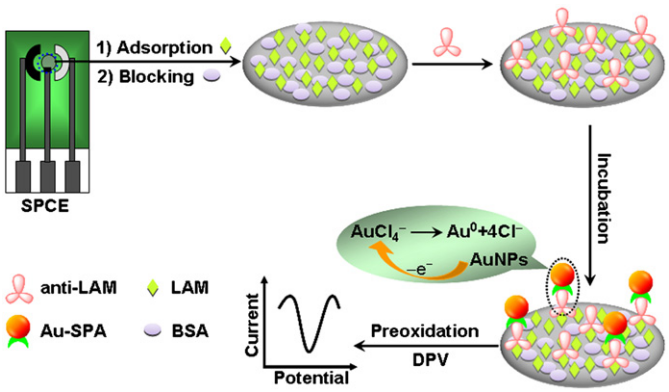

Scheme 1. Schematic representation of the preparation of immunosensor and analytical procedure for the detection of anti-LAM.

the anti-LAM antibody for TB diagnosis (Scheme 1). LAM is a polysaccharide which possesses excellent film-forming ability, and can be immobilized onto the rough carbon electrode surface by passive adsorption; the anti-LAM was bound to the LAM for further capture of Au-SPA on the chip. An electrooxidization method of AuNPs in 0.1 M HCl was applied to produce electroactive $AuCl_4^-$ for differential pulse voltammetry (DPV) detection (Idegami et al., 2008). Although AuNPs as electrochemical label for signal enhancement have already been reported in our previous work (Leng et al., 2010), there are two advantages as follows. One is that antibody-antigen in this approach is without any further modification and the other is that Au-SPA as a universal signal tag can be widely applied in the detection of all kinds of other antibodies. The immunosensor showed a good performance with high sensitivity, selectivity, and simple operation. In comparison to the enzyme-based immunosensor, AuNPs as signal tag exhibited many advantages such as no requirement of deoxygenation, high stability and so on. The enzyme-free signal amplification strategy provides a useful avenue to construct an ultrasensitive assay and low-cost chip for early diagnosis of TB.

2. Experimental

2.1. Materials and reagents

Lipoarabinomannan and rabbit anti-LAM polyclonal antibody were purchased from Dongguan Hannuo Biological Technology Ltd. Gold nanoparticles labeled Staphylococcal protein A was purchased from Shanghai Xinran Biological Technology Ltd. Bovine serum albumin (BSA) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, USA). All other reagents were of analytical grade and used without further purification. 50 mM phosphate-buffered saline (PBS; pH 7.4) was prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4. PBS (50 mM, pH 7.4) containing 10 mg mL^{-1} BSA was used as the blocking buffer. All aqueous solutions were prepared using ultra-pure water ($\geq$ 18 MΩ, Milli-Q, Millipore).

2.2. Apparatus

Preoxidation of AuNPs bound on the immunocomplexes followed by DPV measurement was automatically performed on a CHI 660C electrochemical workstation (CH Instruments, Inc., USA). Scanning electron microscopic (SEM) images were obtained using a Hitachi S-4800 scanning electron microscope (Hitachi, Japan).

2.3. Preparation of immunosensor

According to the previous literature (Yu et al., 2004), a three-electrode SPCE system with graphite working electrode (2.0 mm in diameter), graphite auxiliary electrode, and Ag/AgCl reference electrode was fabricated with the following steps. First, a layer of silver ink was screen-printed on the surface of a nylon sheet to act as a conductive band. Second, a layer of graphite was imprinted to cover the silver film except for the area that served as the reference electrode which was oxidized electrochemically in KCl solution to obtain the Ag/AgCl reference. Finally, the conductive bands were insulated by overlaying a silicone rubber layer to expose the conjunction tips and the three-electrode areas.

1 μL of LAM at 0.4 mg mL^{-1} in water was dropped on the working electrode which was rinsed by ultra-pure water before use. The chip was incubated in a water vapor saturated environment at 4 °C overnight to allow the passive adsorption of antigens onto the carbon electrode surface. After incubation, excess antigens were rinsed with ultra-pure water. For suppression of unspecific adsorption, the electrochemical microcell was incubated with 50 μL blocking buffer for 40 min at room temperature. After rinsing thoroughly with ultra-pure water, the resulting immunosensor was stored at 4 °C prior to use.

2.4. Immunoassay procedure

A 5 μL sample containing anti-LAM was used to incubate the LAM modified SPCE for 30 min at room temperature. The immunosensor was then washed with ultra-pure water and incubated with 5 μL of 10 μg mL^{-1} Au-SPA. After the sensor was washed with ultra-pure water, 50 μL 0.1 M HCl was dropped on the electrochemical microcell to perform the electrooxidation of AuNPs at a constant potential of +1.3 V for 30 s, immediately followed by DPV detection from +0.6 V to 0 V, with a step potential of 4 mV, a pulse amplitude of 50 mV, and a pulse period of 0.2 s.

3. Results and discussion

3.1. Characterization of fabrication of biosensor

The surface morphology of the electrode is a vital factor affecting the immunosensor performance. Fig. 1A and B shows the morphologies of bare and LAM modified SPCE by SEM. The surface of bare SPCE composited with graphite powder (50 nm in diameter) (Fig. 1A) was porous so that it could increase the surface area for large loading of antigens and accelerate the diffusion of analytes in the formed cavities on the electrode surface. After the immobilization of LAM (Fig. 1B), a homogeneous and smooth film on the SPCE surface was formed due to the excellent film-forming ability of LAM. The mushy configuration reveals that LAM was firmly attached to the surface of SPCE, leading to good fabrication stability of the immunosensor.

3.2. Optimization of conditions for electrochemical detection

The concentrations of LAM solutions were optimized in order that a maximum number of antibodies would be bound to capture the Au-SPA as an electrochemical tag on the working electrodes for signal amplification. The working electrodes were coated with different concentrations of antigen solutions, followed by blocking with 1% BSA and binding with 1 mg mL^{-1} antibodies and 10 μg mL^{-1} Au-SPA tag. The peak currents increased with increasing LAM concentrations until the concentrations reached 0.4 mg mL^{-1} (Fig. 1C). Therefore, 0.4 mg mL^{-1} of LAM solution was chosen for coating the SPCE.

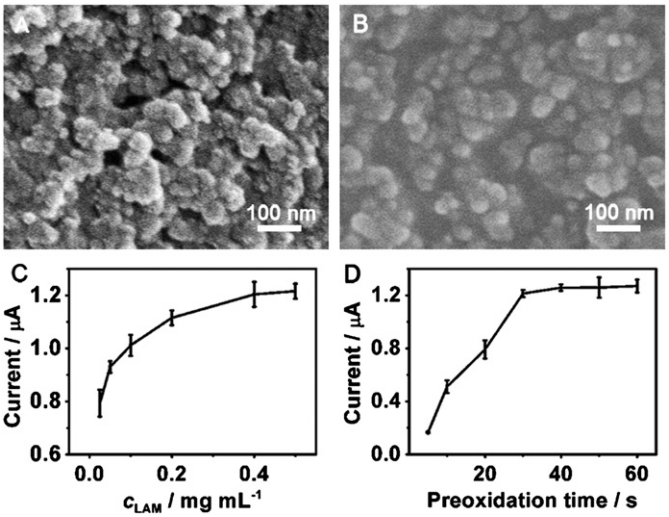

Fig. 1. SEM images of bare (A) and LAM modified (B) SPCE, and optimization of the coating antigen (C), and preoxidation time (D) on DPV responses to 10 μg mL^{-1} anti-LAM.

The electrooxidation of AuNPs in HCl and strong adsorption of the produced $AuCl_4^-$ on the rough carbon surface enable direct electrochemical detection of the amount of captured gold for signal amplification. For obtaining the enhanced electrochemical signal, the conditions for electrooxidization of the captured AuNPs should be optimized. Preoxidation potential must be high enough to oxidize AuNPs in HCl to $AuCl_4^-$ but not too high potential which would cause the damage of the SPCE and decrease the electrochemical signal. The peak currents increased with the increasing of preoxidation potential from 1.2 to 1.3 V, and reached the maximum value at the preoxidation potential of +1.3 V. When the potentials were beyond +1.3 V, the peak current decreased greatly and showed poor repeatability. Therefore, +1.3 V was chosen as the preoxidation potential (Leng et al., 2010). At the preoxidation potential of +1.3 V, the oxidation time was important as well. An insufficient time would lead to the incomplete oxidation of AuNPs, and redundant time would not increase the electrochemical signal; while $AuCl_4^-$, the preoxidation product, might diffuse away and then decrease the electrochemical signal. As shown in Fig. 1D, the DPV responses tended to a stable value at the time of 30 s. Therefore, 30 s was chosen as the preoxidation time, showing a feasibility to implement a rapid detection.

3.3. Performance of the immunosensor

Under optimal detection conditions, the electrochemical response depends on the formation of immunocomplex on the electrode surface. A standard solution of anti-LAM at a known concentration was added into the incubation solution to form the immunocomplex, and then bound with Au-SPA through interaction between the helices I and II of SPA and the Fc region of immunoglobulin (IgG) of anti-LAM without affecting the antibody binding site (O'Seaghdha et al., 2006). Moreover, full length SPA has a binding stoichiometry of two IgG molecules per molecule of SPA, which could enhance the loading of AuNPs for signal amplification. The captured AuNPs on the immunosensor surface can be preoxidized at +1.30 V for 30 s, and then reduced to produce the detectable electrochemical signal without deoxygenation. As expected, with increasing concentrations of anti-LAM the peak currents increased, which resulted from the increasing amount of Au-SPA captured on the immunosensor (Fig. 2A). The calibration plot shows a good linear relationship between the peak current and

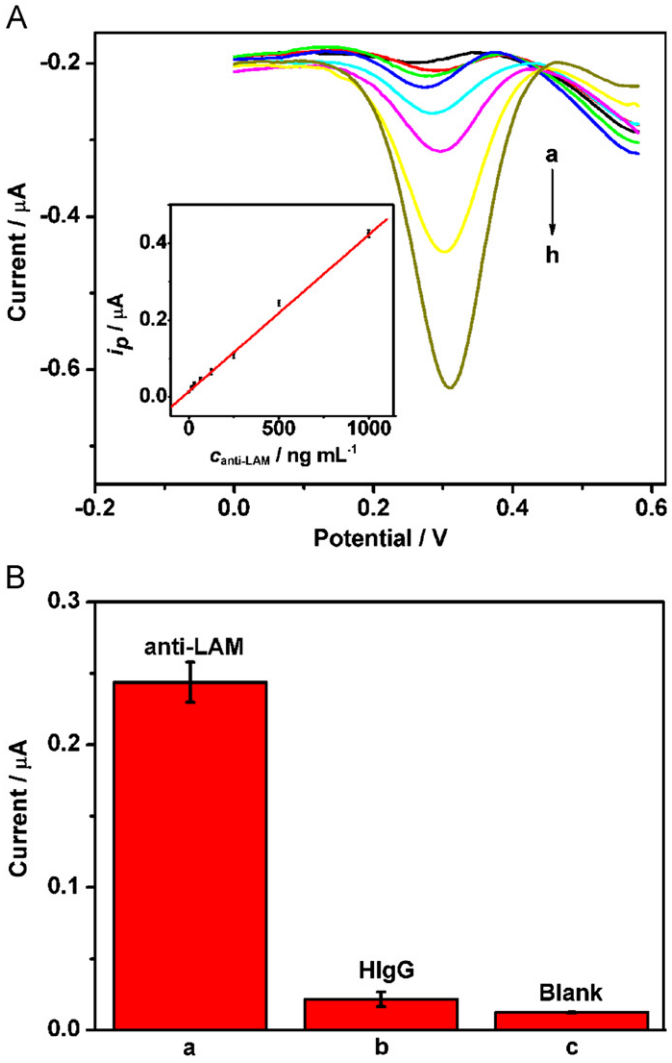

Fig. 2. (A) DPV responses for the detection of anti-LAM using Au-SPA at anti-LAM concentrations of 0, 15.6, 31.2, 62.5, 125, 250, 500, and 1000 ng mL^{-1} from (a) to (h) in order. Inset: corresponding calibration curve. (B) DPV responses of immunosensors to 500 ng mL^{-1} anti-LAM (a), 10 μg mL^{-1} HIgG (b), and blank (c).

the value of the anti-LAM concentration in the range from 15.6 to 1000 ng mL^{-1} with a correlation coefficient of 0.992 (Fig. 2A, inset). The detection limit corresponding to the signals of 3 standard derivations above the mean for a zero standard was 5.3 ng mL^{-1} for anti-LAM. Since the serum anti-LAM level is maintained above 10,000 ng ml^{-1} in TB patients (Sousa et al., 1998), the low detection limit of this method was suitable for the practical application in TB monitoring.

3.4. Selectivity, reproducibility and stability of immunosensor

Many substances in human serum such as human IgG (HIgG) might cause interference when detecting the anti-LAM in TB patients' serum. For the evaluation of HIgG interference, 5 μL HIgG at 10 μg mL^{-1} was incubated on the as-prepared immunosensor, and washed with ultra-pure water, followed by incubation with 5 μL Au-SPA. Compared with that of 500 ng mL^{-1} anti-LAM (Fig. 2B, column a), the reduction peak of the immunosensor in 10 μg mL^{-1} HIgG was only 8.9% (Fig. 2B, column b), which was almost as low as control (Fig. 2B, column c). As a result, the interference of HIgG in human serum had little effect which could be neglected.

The inter-assay precision of the immunosensor was evaluated using five chips. The coefficients of variation for inter-assay were 4.6% and 4.1% for 62.5 and 250 ng mL^{-1} anti-LAM, respectively, indicating acceptable fabrication reproducibility. When the immunosensor was not in use, it was stored at 4 °C. After a storage period of 10 days, the DPV response was 94.7% of initial response to antibodies. Thus the storage stability of the immunosensor was acceptable, exhibiting a potential application in practice.

4. Conclusions

An enzyme-free immunosensor was proposed by employing AuNPs as electrochemical tags for detection of anti-LAM antibody at a disposable chip. The detection of antibodies in early tuberculosis diagnosis showed attractive advantages of the strong antibody response, no requirement of living cells, and noninvasion. The immunosensor was directly prepared by simple adsorption of capture antigen on bare SPCE. The porous surface of SPCE could increase the surface area for large loading of antigens and accelerate the diffusion of the analytes. With a sandwich immunoassay format, the AuNPs were introduced as an electrochemical tag for signal amplification since each AuNP contains thousands of atoms, leading to relatively high sensitivity. Moreover, AuNPs as signal tag exhibited many advantages such as no requirement of deoxygenation, and high stability. Compared with commercial enzyme-linked immunosorbent assay (Ben Selma et al., 2010), the proposed strategy is more rapid and of low cost in the detection of anti-LAM. In addition, the antigen and antibody in our approach are without any further modification; therefore, this presented method is simple and user friendly, and requires little hands-on technical time. The proposed strategy provided a convenient tool and signal amplification platform for the analysis of antibodies, and possessed promising application in clinical TB diagnosis.

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